IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Holger ENGEL *et al.*	Confirmation No.: 8390
Appl. No. 10/669,976	Art Unit: 1637
Filed: September 24, 2003	Examiner: S. K. Mummert
For: **Enhanced Coamplification of Nucleic Acids**	Atty. Docket No.: 00051-0010-001

Declaration of Dr. Dirk Löffert Under 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
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Sir:

1. I, Dr. Dirk Löffert, hereby declare and state as follows:

2. I am one of the named inventors of U.S. Application No. 10/669,976 (hereinafter "the '976 application"), filed September 24, 2003, entitled "Enhanced Coamplification of Nucleic Acids." I am an also employee of the assignee of the '976 application, QIAGEN® GmbH.

3. I hold the degree of Doctor of Philosophy. A recent copy of my Curriculum Vitae, accurately listing my scientific credentials and work experience is attached herewith as Exhibit C.

4. I have read and understand claims 1, 2, 4-22, and 25-37, currently pending in the '976 application. I have also read and understand the Office Action, dated October 26, 2010.

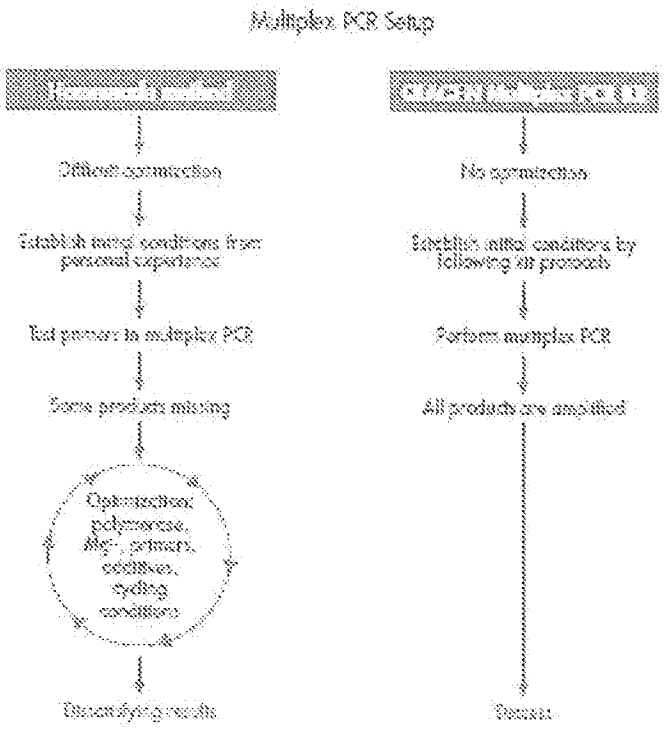
5. The presently claimed invention provides a rapid and efficient method that allows the skilled artisan to conduct a multiplex PCR assay in which six or more target sequences are simultaneously amplified with little or no optimization required. These

unexpected and superior results are discussed in "New QIAGEN® Multiplex PCR Kit" QIAGEN® News 5:13-16 (November 2002) (hereinafter "QIAGEN® News," copy attached herewith as Exhibit A):

> The new QIAGEN® Multiplex PCR Kit is the first kit specifically developed for multiplex PCR. The simple multiplex master-mix solution eliminates the need for lengthy optimization procedures, such as adjusting the amounts of Mg^{2+} and enzyme or even, as frequently required, adjusting primer concentrations. Now standard multiplex PCR applications are fast and easy to perform.

QIAGEN® News at page 13, first column, second paragraph.

6. In QIAGEN® News, the presently claimed methods were compared with standard multiplex PCR methods:



7. The results of the experiments set forth in QIAGEN® News were carried out by technicians working in laboratories in QIAGEN® GmbH. I am familiar with the experiments, and describe below the methodology that was used to generate the results.

8. Reactions representative of what was available in the art at the time of filing the '976 application (hereinafter "standard methods") were carried out using the reaction components shown in Table 1 below. The standard methods utilized a conventional PCR buffer (AMPLI TAQ GOLD® buffer) and enzyme with a chemical hot-start, as described for example in Birch *et al.*, U.S. Patent No. 5,773,258. The amount of $MgCl_2$ was varied to determine the optimal concentration for multiplex PCR.

Table 1: Setup $MgCl_2$ titration with AMPLI TAQ GOLD® Buffer

Component	Final concentration
10x AMPLI TAQ GOLD® buffer (C07612)	1x
$MgCl_2$ (25 mM ABI; C07722)	1.5 mM / 2.5 mM / 3.5 mM
AMPLI TAQ GOLD® (5U/µl; C04799)	2.5 U

dNTP Mix (4mM)	0.4 mM
Primermix 16 plex (2.5 µM each Primer)	0.2 µM
Human genomic DNA	20 ng
H_2O	As needed
Total Volume	50 µl

9. The methods of the presently claimed invention on the other hand were carried out using the reaction components set forth below in Table 2. The QIAGEN® Multiplex PCR Master Mix contains a non-ionic, polymeric volume exclusion agent at an amount such that the final concentration of the volume exclusion agent in the total mixture is from 1 to 20 weight %. The QIAGEN® Multiplex PCR Master Mix also contains a thermostable hot start DNA polymerase.

Table 2: Setup Multiplex PCR

Component	Final concentration
2x QIAGEN Multiplex PCR Master Mix	1x
Primermix 16 plex (2.5 µM each Primer; Table 4)	0.2 µM
Human genomic DNA	20 ng
H_2O	As needed
Total Volume	50 µl

10. Table 4 below shows the primer sequences present in the Primermix 16 plex:

Table 4: Primer 16-plex

Sequence	Name
GCC GAC AAA AGG AGA TCT GTG AGA A	ckit-for
GGC AAT GAC ATA CCA AAG GCT GGT A	ckit-rev
GCA CTG ATG GGC ACT GGA AAA CAT	PRPE3-for
GGA GCC AGA GGT ATC CAG GCA A	PRPE3-rev
TCT TCA ACC TCG CTG TGG CTG A	AGTRII3-for
ATC TTC AGG ACT TGG TCA CGG GTT	AGTRII3-rev
GCA CAA GGT CCC AGC ATC ATT GAT	mb1-for
CCG CGC AGA ACA GGA GGA TGA T	mb1-rev
CCT ATC TTC CTG CTG CTG GAC AA	B29E4-for
GGA GAG GGA TGG AGA TCA GAG TGT TA	B29E5-rev
TTT CCA GAC TTC CTG AGC CCT CAT	CD19E12-for
GCA TAC AGG ATT CCT CTC ATA TCC TCA T	CD19E13-rev
TGC CAT CCT CTT GGT GCT GGT CT	CD40E7-for
CAG CAG TGT TGG AGC CAG GAA GAT	CD40E9-rev
GCTGTTTGATGTCCTGCACGAG	ERCC1-for
GCCTGGCCTGGGAGGACGATT	ERCC1-rev
GAAGTTGTACAGGCCCAGTGTAGGAA	IL17F-for
CTTCTCCAACTGGAAAGAAACAGAGC	IL17F-rev
CTGTGAGCAGCTAGTGGTGGCTTC	Aqua-for
AACCAGTTGCATCATTCCCAGAAC	Aqua-rev
GGACTGCATTACAACAAATTCGGACAC	IL4P-for
CGTTACACCAGATTGTCAGTCACTTGG	IL4P-rev
GCTTGAGCAACCTGGCTAAGATAGAGG	CD59-for
GAGTTAGCAGGAGGCTGGATGCAGATG	CD59-rev
AGTGTGGGGTGCTAAACAGATCTCA	CAS10-for
AACCAAGGTGCAAAACAGTCTGCTA	CAS10-rev
CTTTTGGGAGTGTGGAAGTCCATAA	CD38-for
GGTGGGATCCTGGCATAAGTCTC	CD38-rev
CAACGTCTGCACTCTCGTGAGGG	ELA-for
CGGAGCGTTGGATGATAGAGTCGAT	ELA-rev
TGTCTGCAGTAGAGGTGGAGATCCAT	CD14-for
GCATCTCGGAGCGCTAGGGTTT	CD14-rev

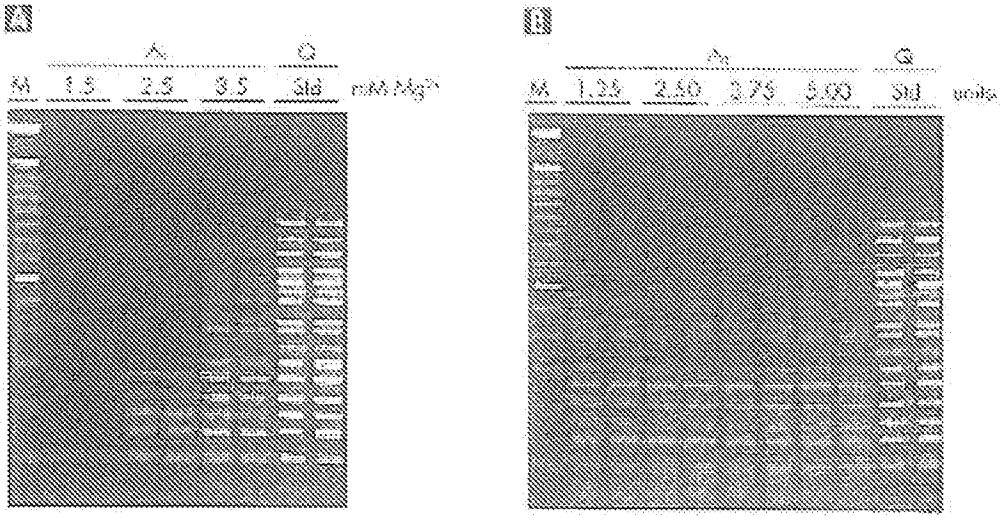
11. For the methods carried out under the presently claimed invention, the difference between the lowest copy number and highest copy number was less than 10-fold.

The standard methods and Multiplex PCR reactions were performed using the thermal cycling conditions given in Table 3 below.

Table 3: Cycling Protocol	
Initial activation step	For QIAGEN® Multiplex Master Mix: 95°C, 15 min / For AMPLI TAQ GOLD®: 95°C, 10 min
Denaturation	94°C 30 sec
Annealing	61°C 90 sec
Extension	72°C 90 sec
Number of cycles	35 x
Final extension	68°C 15 min

12. As noted in Table 3, each of the 35 cycles comprised heating the reaction mixture to denature the strands, priming the denatured strands by cooling to a second temperature, and forming primer extension products.

13. 10 µl of each PCR reaction described in Tables 1-3 was then analyzed on an agarose gel stained with ethidium bromide to detect the primer extension products. The results of the gel analysis are provided below in Figure 1A of QIAGEN® News.



Multiplex PCR of 16 targets (99–955 bp) was carried out for 35 cycles using standard conditions (Lab) for the QIAGEN Multiplex PCR Kit (Q) without further optimization or using a variety of conditions with a hotstart DNA polymerase from Supplier A (A). A Comparison using 2.5 units/50 µl reaction of the hotstart DNA polymerase from Supplier A and with the indicated Mg^{2+} concentrations. B Comparison using the optimized Mg^{2+} concentration (3.5 mM) for the hotstart DNA polymerase from Supplier A, and the indicated amounts of enzyme per 50 µl reaction. M: marker.

14. As shown in Figure 1A, varying magnesium ion concentration (e.g., 1.5, 2.5, 3.5 mM) in the standard methods did not result in the successful co-amplification of the 16 targets in detectable quantities (see lanes 2-7). In contrast, using the methods of the presently claimed invention, successful co-amplification was achieved **in the first attempt** (see lanes 8 and 9 "Q").

15. The methods described above were repeated utilizing a fixed amount of $MgCl_2$ (3.5 mM), and varying the amount of AMPLI TAQ GOLD® for the standard methods (e.g., 1.25, 2.5, 3.75, 5 Units). The reaction components are set forth in Tables 5 (standard methods) and 6 (methods of the presently claimed invention) below.

Table 5: Setup AMPLI TAQ GOLD® titration with AMPLI TAQ GOLD® Buffer	
Component	Final concentration
10 x AMPLI TAQ GOLD® Buffer (D01345)	1x
$MgCl_2$ (25 mM ABI; D00424)	3.5 mM
AMPLI TAQ GOLD® (5U/µl; CO4799)	1.25 U / 2.5 U / 3.75 U / 5 U

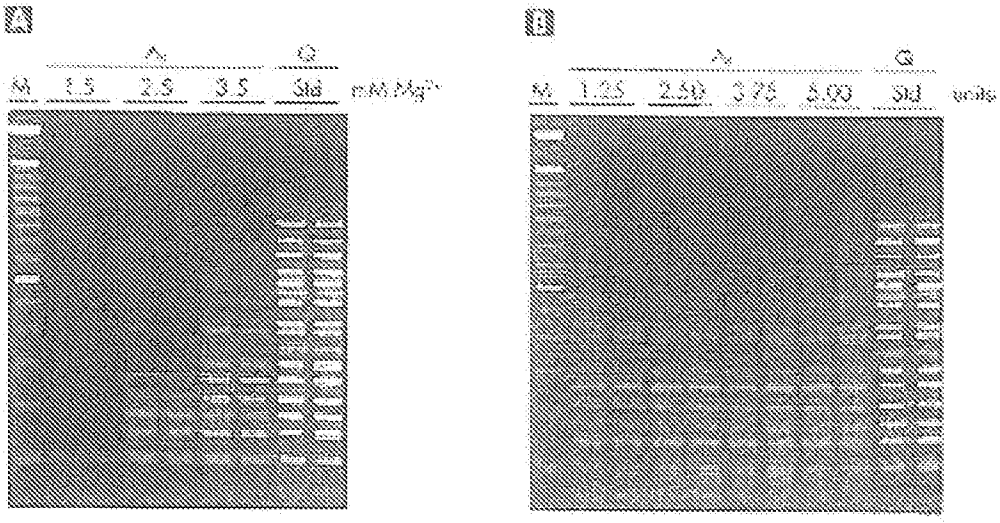
dNTP Mix (4mM)	0.4 mM
Primermix 16 plex (2.5 µM each Primer)	0.2 µM
Human genomic DNA	20 ng
H_2O	As needed
Total Volume	50 µl

Table 6: Setup QIAGEN® Multiplex PCR MM	
Component	Final concentration
2x QIAGEN Multiplex PCR Master Mix	1x
Primermix 16 plex (2,5 µM each Primer; Table 4 above)	0.2 µM
Human genomic DNA	20 ng
H_2O	As needed
Total Volume	50 µl

16. PCR was performed using the thermal cycling conditions given in Table 7.

Table 7: Cycling Protocol	
Initial activation step	For Qiagen Multiplex Master Mix: 95°C 15 min / For AmpliTaq Gold 10 min
Denaturation	94°C 30 sec
Annealing	61°C 90 sec
Extension	72°C 90 sec
Number of cycles	35 x
Final extension	72°C 10 min

17. 10 µl of each PCR reaction was analyzed on an agarose gel stained with ethidium bromide. The results are shown in Figure 1B of QIAGEN® News.



Multiplex PCR of 16 targets (99–955 bp) was carried out for 35 cycles using standard conditions (Std) for the QIAGEN Multiplex PCR Kit (Q) without further optimization or using a variety of conditions with a isolated DNA polymerase from Bacillus A1 (A). A Comparison using 2.5 units/50 µl reaction of the isolated DNA polymerase from Bacillus A1 and with the indicated Mg^{2+} concentrations. B Comparison using the optimized Mg^{2+} concentration (3.5 mM) for the isolated DNA polymerase from Bacillus A1 and the indicated amounts of enzyme per 50 µl reaction. M marker.

18. As shown in lanes 2-9 of Figure 1B, optimization of the enzyme concentration with ostensible "optimized" magnesium ion concentration (see above) also failed to allow the standard methods to successfully coamplify the 16 targets in detectable quantities. In contrast, the methodology of the presently claimed invention, the results of which are represented in lanes 10-11 of Figure 1B, allowed for successful co-amplification **in the first attempt**.

19. As opposed to the "tedious" standard methods which "require extensive optimization" often leading to "disappointing" results (*see* QIAGEN® News, p. 14, left col.), the presently claimed methods "eliminate the need for lengthy optimization procedures" and are "fast and easy to perform." (*See* QIAGEN® News, p. 13, left col.). Indeed, the presently claimed methods require "no optimization" (*see* QIAGEN® News) and "tedious optimization procedures are virtually eliminated." *See* "Highly Efficient Multiplex

PCR Using Novel Reaction Chemistry," Agilent Technologies ©2003 at page. 4, left column. ("Engel," Exhibit B).

20. It is my opinion that those in the field would have determined that in order to obtain results similar to those of the presently claimed invention, substantial optimization of standard methods would have been required, with no guaranty and no reasonable expectation of arriving at the surprising and unexpected results that are shown in lanes 8/9 and 10/11 if Figures 1A and 1B, respectively, of QIAGEN® News, described above.

21. Thus, the presently claimed methods clearly display unexpected and surprising results, as compared to standard methodology known at the time of filing the present application.

22. I further state that all statements made on my own knowledge are true and that all statements made on information and belief are believed to be true and further that willful false statements and the like are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the U.S. Code and may jeopardize the validity of the application or any patent issuing thereon.

08. APR. 2011
Date

Dr. Dirk Löffert